In vitro investigation of the inhibitory effect of fucoidan on α-glucoisidase enzyme activity

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Feburary 2013

Table of Contents

Chapter 2 Manuscript

List of figures

List of abbreviations

APTT	Activated partial thromboplastin time
CVDs	Cardiovascular diseases
DNA	Deoxyribonucleic acid
FAs	Fatty acids
IGT	Impaired dependent diabetes
K_m	Michaelis constant
LMWF	Low molecule weight fucoidan
mg	Milligram
ml	Milliliter
NF	Native fucoidan
NO	Nitric oxide
OSF	Oversulfated fucoidan
PNPG	4-Nitrophenyl β-D-glucuronide
PUFAs	Polyunsaturated fatty acids
RNA	Ribonucleic acid
STOP-NIDDM	Non-Insulin Dependent Diabetes Mellitus
V_{max}	Maximum reaction rate
µg	Microgram

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Abstract

Introduction. Diabetes mellitus, a dysglycaemic disorder is causing a huge financial budget to society, government agencies and individuals every year. Diabetic patients are at the risk of long-term macro– and microvascular damages. Current therapies for diabetes include lifestyle modification and pharmacological interventions. However, conventional oral anti–hyperglycemic drugs produce a range of side effects such as hypoglycemia; therefore safer and more effective medications are required to manage diabetes and prevent the long-term complications. Fucoidan represents a large class of highly sulfated and water-soluble polysaccharides presents in all of the brown algae. Its antidiabetic effect has been reported in the literature. The objective of this study was to investigate the inhibitory effects of three different types of fucoidan on the activity of intestinal enzyme, α-glucosidase, which is involved in the facilitation of glucose absorption.

Methods. α–glucosidase enzyme was extracted from the rat–intestinal acetone powder. The enzyme activity of α–glucosidase was determined using an enzyme substrate 4-Nitrophenyl β-D-glucuronide (PNPG) and enzyme extract. The enzyme kinetics of α–glucosidase was performed using different concentrations of substrate. The calculation of V_{max} and K_m were carried out based on Lineweaver–Burk plot. The inhibitory effect of three different types of fucoidan (*Undaria pinnatifida*, *Fucus vesiculosus*, *Fucus synergy*) was investigated. The stock solution containing 1000 μg ml^{-1} of fucoidan was prepared. Different concentrations of fucoidan solutions were prepared by serially diluting the stock solution. Acarbose and Milli–Q water were used as positive control and negative control respectively. In enzyme assay, a reaction mixture contained enzyme extract, substrate and fucoidan.

Results and discussion. The activity of extracted α–glucosidase was found to be 0.0381 U ml^{-1}. The V_{max} and K_m values were 0.2885 μg min^{-1} and 8.8339 μg ml^{-1} respectively. In the

inhibitory assay, the maximum percentage of inhibition of acarbose was 61.11%. In contrast, the maximum inhibitory effects of fucoidan extracted from *Fucus synergy* was 7.57%, followed by the extracts of *Fucus vesiculosus* (6.14%) *and Undaria pinnatifida* (3.30%). **Conclusion.** Three different types of fucoidan investigated in the current study had negligible inhibitory effect on the activity of on α–glucosidase enzyme.

Chapter 1 Literature Review

1.1 Marine algae and discovery history in Australia

Marine algae, also known as seaweed is a wide group of organisms which require oxygen and light to produce nutrition for growth (1). Multiple taxonomical methods such as kingdom-division-class axis are developed to classify these organisms because it is impossible to place all of the existing algae into specific categories (2). However, the most common used category is based on their colour, namely phaeophytes (brown algae), rhodophytes (red algae), chlorophytes (green algae) (3) and cyanophytes (blue-green algae) (4). The extracts obtained from marine algae have various therapeutic functions, for instance, anti–tumor, antiviral and anti–coagulant activities (5).

The earliest description about Australian marine algae was made by a scientist from New South Wales as *Conferva umbilicata* in 1800 BC, which is currently known as *Microdictyon umbilicatum*. After that, Captain Vancouver collected marine algae systematically in his expedition in 1791. Thirty–nine years later, Charles Fraser became the first Australian who collected marine algae along the Swan River in Western Australia. Evidence has revealed that the Southern Australian region, including Tasmania is rich in marine algae resources, probably due to favorable growth conditions (6).

1.2 The worldwide consumption, cultivation and utilisation of marine algae

According to statistics reported in 2009, seaweeds contribute to approximately 25% of daily utilisation, including food consumption, agricultural field and health care industry (7) . The annual consumption is dominated by a large portion of brown algae (66.5%), followed by figures of red algae (33%) and green algae (5%) (8). Interestingly, nearly 90% dry seaweed products just come from six countries, namely Chile, Korea, Japan, China, United Kingdom and France (9). Nevertheless, the consumption of seaweed products differs significantly from

Asia to Western countries. By comparison, seaweeds are not authorised as food in European countries until 1990 (10). With the epidemic requirement of seaweed products, artificial cultivation becomes the most productive way because of the high profits and ease of growth (11).

1.3 Nutritional content and potential effects of marine algae

Thirty years ago, the principal use of seaweeds was dominated by various industrial applications, for example, being used as gelling and thickening agents. Polysaccharide extracts such as alginate, carrageenan and agar did not used as food supplement until the year of 1990 in Europe. France was the first country to legislate them for human consumption (10). So far marine algae have been proved to be rich in various nutritional components such as vitamins, proteins, dietary fibres and minerals, and also have shown great interests in nutritional area. Undoubtedly, they are one of the promising food sources to cope with the shortage of food supplementation.

1.3.1 Protein and nitrogen

Generally, high protein and nitrogen content of marine algae is more important in food and agricultural industries (1). For example, some species show an increasing value for flavour formula because of high content of aspartate and glutamate (4). However, a species difference is observed for this aspect. Typically, brown seaweed contains relatively smaller proteinic percentage (average 5~15% of the dry weight), in comparison with that of red and green seaweed which are as high as 10~30%. Overall, marine algae are rich in proteins while poorer in digestibility (12). Although there are no substantial evidences indicating the digestibility of algal proteins, proteolytic enzymes such as pepsin and pancreatin are assumed to be responsible for the degradation of algal proteins (6,15,16).

1.3.2 Fatty acid

Lipid profiles of marine algae suggest that seaweeds have potential health benefit and turn out to be an option for hyperlipoidemic patients' diets (13). Seaweeds contain many essential fatty acids which are good for a balance diet, while low in lipid composition (between 1.5% and 4% of dry matter) (14). Marine algae have a remarkable level of polyunsaturated fatty acids (PUFAs) such as omega 3 (n-3) and omega 6 (n-6) fatty acids (FAs) and function well in preventing or modulating coronary heart disease. Some essential dietary fatty acids, for example, linoleic acids, are also detected in the seaweeds (15).

1.3.3 Polysaccharide and dietary fibres

Polysaccharides exist in many kinds of marine algae and express heterogeneous structures (16, 17). Algae polysaccharides can be divided into three major classes according to their functions and locations. The most common class is storage polysaccharides such as laminarin in brown seaweeds and starch in green seaweeds (16). Another common class is called cellular mucilage polysaccharides and other minor polysaccharides are found on the cell wall, for instance, carrageenans extracted from red seaweeds (14). Cell-wall polysaccharides have been proved to have various medicinal properties due to their sulfated groups (5, 20, 21). Most algae polysaccharides are proposed to be dietary fibre because there is a lack of digestive enzymes in human intestine; thus they cannot be considered as a reliable energy source (14). However, dietary fibre extracted from seaweeds contributes to the nutritional meal replacement for over-weight patients. Fucoidan, known as sulfated fucans, is an important member of sulfated polysaccharides and exhibit many important biological properties.

1.3.4 Minerals and vitamins

The mineral content of marine algae varies with growth environment, seasons, species and manufacturing methods. Generally, the composition of minerals is as high as 40%, containing various essential minerals, such as potassium, calcium, sodium, magnesium, phosphate, and a remarkable percentage of iodine (1, 12, 18). Therefore, edible marine algae have potential to be a prospective source of mineral supplementation product.

In addition to protein, dietary fibres and minerals, seaweeds are also considered to be a good source of vitamins. For example, red and green seaweeds contain significant levels of vitamin A and B (19), while part of the brown seaweeds are potential sources of vitamin C (12).

1.4 Fucoidan

1.4.1 General structure and physicochemical properties

Brown seaweeds produce multiple branched polysaccharides with species–specific sugar substituents. Algal fucoidans represent a family of highly sulfated and water-soluble polysaccharides presenting in all of the brown algae. They compose up to 25–30% of the seaweed dry weight. The biosynthesis pattern and the species diversity contribute to the heterogeneous structures of fucoidan (17, 20). Recent findings suggest that the backbone of fucoidan is constituted to α–(1$\rightarrow$3) linked fucose with substantial sulfate ester and L-fucose groups on C–2 or C–4 position (21). However, fucoidan structure is difficult to be analysed precisely due to the variability of linkage types and species deviation generally.

1.4.2 Structure–biological activities relationship

The algal fucans have some repeating pattern but are heterogeneous with the time of collection and species difference. It has been reported that their biological activities are

highly related with their structures. The position and amount of sulfated groups are two significant parameters that determine the biological activities of fucoidan (1, 22). It has also reported that molecule weight and size of fucoidan contribute to qualitative difference in previous biological studies (5, 25). For example, low molecule weight fucoidan (LMWF) (15-20 kDa) has a pro-angiogenic effect and better anti–coagulant outcomes than those in large size. Therefore, enzymatic degradation methods are used in the manufacturing process, aimed to produce a LMWF while retaining the maximum amount of sulfated groups (5).

1.4.3 Different biological activities of the brown seaweed fucoidans

One consequence of the heterogeneous structures of fucoidan is that each unique structure has a potential to be recognised by a specific receptor (5). Even though mechanisms by which fucoidan insert their biological actions remain controversial due to the complex structures, studies have indicated that fucoidan has considerably valuable therapeutic effects such as anti–coagulant (23, 24), anti–tumor (25), anti-inflammatory (16), antiviral (26, 27) and antioxidation (17).

1.4.3.1 Anti–coagulant and anti–thrombotic activity

Fucoidan has structural similarity in sulfated content with heparin, thus it exhibits anti–coagulant activity. A study that used activated partial thromboplastin time (APTT) mode has shown that fucoidan exhibits strong inhibitory effect on thrombin activity of platelet (28). Some studies illustrate that therapeutic outcomes and mode of action vary with the structure of fucoidan. For instance, increase in the sulfate content can improve the anti–coagulant activity by between 10 and 19% (21). Moreover, straight sulfated fucoidan show indirect inhibitory effect on thrombin while highly branched types can inhibit the activity of thrombin

without other factors (28). Because of the anti–coagulant activity of fucoidan, it can be considered as a promising alternative to heparin.

1.4.3.2 Anti–virals

In recent years, both *in vitro* and *in vivo* studies have demonstrated the antiviral activities of sulfated polysaccharides such as fucoidan. Fucoidan has an interesting function of anti RNA and DNA viruses (1). It has been reported that fucoidan could have protective antiviral activity via direct inhibition of viral replication and activating the host immune defense system (21).

1.4.3.3 Anti–tumor and anti–oxidant activities

Fucoidan is suggested to be a promising agent for anticancer and anti–tumor therapies. Both native and oversulfated fucoidans have been investigated. The results have shown that increasing the extent of sulfation can enhance the inhibition of tumor cell growth through stronger suppression on angiogenesis (29). Besides of its anti–tumor activity, fucoidans extracted from edible seaweeds are effective anti–oxidants.

1.4.3.4 Anti-inflammatory activity

The anti-inflammatory activity of fucoidan has also been investigated. Some extracts of fucoidan can decrease the neutrophil extravasation and transmigration at a dose of 15 mg/kg in an acute peritonitis rat model (28). The inhibitory effect of fucoidan on leukocyte recruitment in the inflammatory model may be associated with the release of nitric oxide (NO) (21).

1.4.4 Toxicity of fucoidan

Development and application of fucoidan as therapeutic agent has been an important research topic. However, data on its safety profile obtained from *in vitro* and *in vivo* studies of fucoidan are relatively limited. Two available research articles (30, 31) have reported the toxicities associated with fucoidan administration. Fucoidan extracted from *Undaria pinnatifida* did not cause genotoxicity when the oral dose was lower than 1000 mg/kg body weight per day in the tested animals. Whereas for fucoidan extracted from *Laminaria japonica* did not show signs of toxicity unless the dose was higher than 900 mg/kg body weight per day in the tested animals. The administration of fucoidan is considered to be safe when given at therapeutic doses. Nevertheless, the long-term side effects and toxicities associated with fucoidan require further investigation.

1.5 Diabetes

The incidents of diabetes mellitus, a dysglycaemic disorder is increasing world widely, causing a huge financial burden to society and individuals every year. According to the latest annual report of Intentional Diabetes Federation, at the end of 2011, there are more than 366 million or 8.3 % of global adult population been diagnosed with diabetes, and the figure is predicted to be double at the end of 2030 (32). Diabetes is a metabolic disease characterized by high blood glucose level with the fasting glucose level more than 140 mg/dl (33). Diabetic patients are at the risk of long-term macro- and microvascular damages that can lead to further severe complications such as blindness, coronary artery disease and renal failure (34, 35).

Diabetes is classified into four main categories, namely type 1 or insulin-dependent diabetes, type 2 or non-insulin-dependent diabetes, gastrointestinal diabetes and other specific types

such as impaired glucose tolerance (IGT) (33, 35). Current antihyperglycaemic treatments only focus on controlling exacerbation and such available approaches have a range of adverse effects such as hypoglycemia, weight gain and nausea(36). Therefore, it is of importance to develop new antidiabetic therapies that could provide more efficient therapeutic outcomes with fewer side effects. In particular, decreasing the postprandial glucose levels through retarding the conversion of carbohydrates, fat and proteins into glucose is considered to be a promising approach because this method has a lower risk of hypoglycemia and weight gain which are frequently seen with the administration of other anti-diabetic agents. Nowadays, the inhibitory effects of natural products on digestive starch enzymes, for example, α-glucosidase, have drawn much attention in this aspect (37).

1.5.1 Carbohydrate metabolism and postprandial hyperglycaemia

Plasma glucose level is tightly controlled within a narrow range in nondiabetic people. After taking meals, increase in the postprandial glucose stimulates the secretion of insulin from pancreas β–cells (38). The balance between the rate of carbohydrate absorption from the gastrointestinal tract and the rate of utilisation is determined by several parameters such as nutrient type, digestive enzymes and reaction time of peripheral tissues to insulin secretion (39). In diabetic patients, postprandial hyperglycaemia could be observed as a consequence of insulin resistance or insulin deficiency (40). Evidences have suggested that postprandial hyperglycaemia itself is a significant risk factor of cardiovascular diseases (CVDs) and contributes to mortality rate (34). Studies have demonstrated that the risk of myocardial infarction is two to three fold greater in patients who have developed postprandial hyperglycaemia, compared with those with normal glucose tolerance (39). CVDs are associated with a soaring level of mortality in the patients with impaired dependent diabetes (IGT) and type 2 diabetes (34, 41).

1.5.2 Currently available treatments of diabetes

The main purpose of diabetic therapies is to control the blood glucose level and to prevent the complications such as CVDs (24, 29, 31). Lifestyle modification which involves dietary changes and exercise becomes a key strategy of prophylaxis. However, this approach alone is not sufficient for long-term glycaemic management. Therefore, pharmacological intervention is required to maintain the treatment outcomes (34). There are three main types of oral antidiabetics, namely insulin secretagogues, insulin sensitisers and inhibitors of carbohydrate absorption (38).

Recently, inhibitory effect of both synthesised and natural therapies on starch digesting enzymes such as α–glucosidase have been investigated since inhibiting the rate of carbohydrate absorption provides a safe way, with a lower risk of hypoglycaemia and greater benefits on preventing diabetic complicates than conventional medications (36).

1.5.2.1 Synthetic α–glucosidase inhibitors: acarbose and miglitol

The development of α–glucosidase inhibitors such as acarbose and miglitol has provided a new approach in managing diabetes. Basically, these molecules reversibly bind to the carbohydrate region of α–glucosidase (an intestinal enzyme) This would reduce the digestion of carbohydrates and hence decrease the intestinal absorption of glucose (24, 28, 32). The major side effects of α–glucosidase inhibitors is gastrointestinal disturbance such as diarrhoea and abdominal discomfort, which are diminished with reduced dose (39). Acarbose and miglitol are two common drugs that can inhibit the activity of α–glucosidase enzyme. Diarrhoea, flatulence and abdominal distension are the major reported side effects of acarbose and miglitol. Large randomized clinical studies such as Non-Insulin Dependent Diabetes Mellitus (STOP-NIDDM) randomised trial (42, 43) and randomized double-blind

trial (44) have reported that both acarbose and miglitol showed superior outcomes in preventing postprandial hyperglycaemia and CVDs on patients with IGT or established type 2 diabetes, compared with insulin secretagogues and insulin sensitisers. Unlike the use of sulphonylureas and glinides, both of which are widely used for management of type 2 diabetes, administrating acarbose or miglitol alone is not associated with weight gain and hypoglycaemia (34, 45). Additionally, acarbose and miglitol are safer to use with metformin and other oral antihyperglycaemic agents due to less drug-drug interactions (33, 35).

1.5.2.2 Natural α–glucosidase inhibitors from plants and marine algae

Other alternative sources for α–glucosidase inhibitors are plants and marine algae. The active extracts from plants and seaweeds provide more flexible food consumption choice for diabetic patients. Studies have shown that some plants extracts have hypoglycemic effects (37, 46, 47). For example, phenolic constituents of the heartwood of *Pterocarpus marsupium* can decrease the blood glucose level significantly in diabetic rat, in comparison of metformin (46). The anti–diabetic effect of both oversulfated fucoidan (OSF) and native focidan (NF) are also assessed via enzymatic reaction kinetics and their inhibitory activity against starch digestive enzymes (47). The results illustrated that the NF had little inhibitory effect on α–amylase while OSF can reduce the starch digestibility by 12%. Since fucoidan has a large molecular weight and low digestibility, it is less likely to be absorbed from the gastrointestinal tract. Hence, one possible mechanism of action for its potential anti–diabetic effects could be inhibition of intestinal enzymes.

1.6 Objectives

The aim of this study was to investigate the inhibitory effects of fucoidans from *Undaria pinnatifida*, *Fucus vesiculosus* and *Fucus synergy* on the activity of α-glucosidase.

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Chapter 2 Manuscript

In vitro investigation of inhibitory effect of fucoidan on α-glucoisidase enzyme activity

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Key words: fucoidan, acarbose, α–glucosidase, inhibitory assay

Abstract

Context. Diabetes causes a significant burden to the society and individuals. Current treatments exhibit many limitations. Therefore more effective and safer medications are required for its treatment.

Objective. The aim of this study was to investigate the inhibitory effects of fucoidans from *Undaria pinnatifida, Fucus vesiculosus* and *Fucus synergy* on the activity of α-glucosidase.

Materials and methods. Enzyme activity and enzyme kinetics of α–glucosidase enzyme extracted from rat–intestinal acetone powder were carried out using standard calibration curve of p–nitrophenol. Then enzyme inhibition assay was carried out using fucoidan from three different sources. Water and acarbose were used as negative and positive controls respectively.

Results. The enzyme activity, K_m and V_{max} of α–glucosidase were 0.0381 U ml^{-1}, 8.8339 μg and 0.2885 μg ml^{-1} respectively. Acarbose showed inhibition of α–glucosidase activity while three different types of tested fucoidan showed very little or no activity.

Discussion and conclusion. Experimental results showed negligible inhibitory effect of fucoidan on the activity of α–glucosidase.

Introduction

Diabetes is a group of heterogeneously metabolic disorders, characterised by hyperglycemia and glucose intolerance. It is mainly divided into insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). Destruction of pancreatic beta-cells contributes to type I diabetes (IDDM), whereas insulin resistance or relative insulin deficiency is believed to be one major cause of type 2 diabetes (NIDDM) (1). Long–term abnormal glucose metabolism in diabetes provokes damage to blood vessels and double the risk of cardiovascular diseases (CVDs). The diabetic complications could be both macrovascular and microvascular, such as ischemic heart disease and blindness (2). Generally, the elderly patients with long–term poor blood glucose control are more likely to develop nephropathy, retinopathy, neuropathy and CVDs. By the end of 2000, 3% of the Western population had non–insulin–dependent diabetic mellitus (NIDDM) and 40% of these were patients over 60 years old. (3). Among these complications, CVDs such as coronary heart disease, stroke and peripheral vascular disease are the main reasons of mortality in diabetic patients. Many studies have indicated that postprandial hyperglycemia together with other risk factors, for example, smoking contribute to the development of CVDs (4). Therefore, preventing the onset of CVDs and correcting the postprandial hyperglycemic state are two core strategies in diabetic treatment. Current approaches include lifestyle modification and medication intervention (5). However, current pharmacological therapy is still facing a great challenge because evidence shows that some conventional oral anti-hyperglycemic agents increase the cardiovascular mortality (6). In the recent years, natural

products (7, 8) and chemically synthesised agents targeted on digestive starch enzymes, for example, α-glucosidase, have proven to be effective in treating postprandial hyperglycemia. Acarbose, a synthesised α-glucosidase inhibitor exhibited promising antidiabetic effect. It not only reduces postprandial hyperglycemia but also reduces the risk of CVDs by 49% (9). However, a study in 1994 revealed that approximately 46% of subjects complained about gastrointestinal discomfort such as flatulence and diarrhoea and about 2% did not tolerate acarbose (5). To reduce the side effects associated with synthesised α–glucosidase inhibitors, natural products extracted from marine algae such as fucoidan have been investigated for their potentially inhibitory effect on α-glucosidase.

Marine algae represents a large family of organisms and contains high content of sulfated polysaccharides which exhibit a variety of biological activities such as anti–oxidant (10), anti–viral and anti–bacterial (11), anti–tumor, anti–inflammatory and anti–coagulant (12). Fucoidan is a water soluble sulfated polysaccharide (13). A small in vitro study has indicated that fucoidan can potentially inhibit the activity of α–glucosidase enzyme (14) probably because of structural similarity to acarbose.

Many factors, such as the structure of fucoidan and types of seaweed sources can influence its inhibitory effect on α–glucosidase activity. Some studies also indicate that those factors make it hard to determine the anti–diabetic effect of fucoidan (15, 16). Hence, more research on anti-hyperglycemic activity of fucoidan is still needed. The objective of this study was to investigate the inhibitory effects of fucoidans from *Undaria pinnatifida, Fucus vesiculosus* and *Fucus synergy* on the activity of α-glucosidase.

Methods

Materials

Rat–intestinal acetone powder, sodium carbonate, 4–nitrophenol, acarbose, monobasic potassium phosphate, dibasic potassium phosphate and 4–nitrophenyl α–D–glucopyranoside (PNPG) were purchased from Sigma–Aldrich (New South Wales, Australia). Three different types of fucoidan (*Undaria pinnatifida*, *Fucus vesiculosus*, *Fucus synergy*) were generously donated by Marinova Pty Ltd (Tasmania, Australia).

Extraction of mammalian α–glucosidase

The method described by Mohamed and co–workers was modified for the extraction of α–glucosidase from rat–intestinal acetone powder (17). Briefly, rat–intestinal acetone powder (200 mg) was mixed with ice cold phosphate buffer (4 ml, 50 mM, pH 6.8). The suspension was then sonicated for 45 minutes using ultrasonic bath (Sonorex RK–100, Bendelin Electronic, Berlin, Germany) kept at approximately 0 to 4°C. The resultant mixture was centrifuged (Eppendof centrifuge, model number 5417R, Hamburg, Germany) at 4°C and 13,000 rpm for 30 minutes. The supernatant was then carefully removed and stored at – 20°C for further experiments.

Determination of enzyme activity

The activity of α–glucosidase was determined using PNPG (an enzyme substrate). The reaction between the enzyme and its substrate was initiated by mixing 10 µl of enzyme extract and 20 µl of 1 mM PNPG with 50 µl of 50 mM phosphate buffer pH 6.8. The

enzyme–substrate mixture was then incubated in a Bioer mixing block (MB–102, Hangzhou, China) at 37 °C (500 rpm) for 30 minutes. The reaction was stopped using 50 µl of 0.1 M sodium carbonate. The intensity of colour, which was proportional to the activity of enzyme, was read at 405 nm using a micro–plate reader (Thermo Scientific Multiskan60, Massachusetts, United States of America). A solution of 4-nitrophenol (100 µg ml^{-1}) was diluted with a 50 mM of phosphate buffer to obtain 5 different concentrations (5, 10, 15, 20 and 25 µg ml^{-1}). A standard calibration curve (n=3) was generated by analysing these standards at 405 nm using a microtiter plate reader. Control samples (n=3) were prepared and analysed in a similar way except the enzyme was denatured by placing it in boiling water for 10 minute. The activity of enzyme (U ml^{-1}) was calculated using a following formula:

$$\text{enzyme activity} = \frac{\text{m (product)} \div \text{MW(product)}}{30 \text{ mins} \times 0.01 \text{ ml}}$$

where m (product) is the mass of p–nitrophenol, MW (product) is the molecular weight of p–nitrophenol.

Enzyme kinetics of α–glucosidase

Kinetic study of the enzymatic reaction was carried out using different concentrations of PNPG in the presence of enzyme. A solution (n=3) containing 50 µl of 50 mM phosphate buffer (pH 6.8), 10 µl of enzyme and 20 µl of different concentrations (ranging from 0.5 to 16 µg ml^{-1}) of PNPG was incubated at 37 °C using a mixing block for 30 minutes. The reaction was then stopped by adding 50 µl of 0.1 M sodium carbonate, followed by incubation in mixing block for approximately 1 minute. The intensity of colour was measured

at 405 nm using a microtiter plate reader. Control samples (n=3) were prepared and analysed in a similar way except the enzyme was denatured by placing it in boiling water for 10 minutes. Samples were prepared in triplicate and analysed in duplicate. Lineweaver–Burk plot was used to determine maximum reaction rate ($V_{max)}$ and Michaelis constant ($K_{m)}$ of the enzyme (17, 18).

Inhibitory assay

The inhibitory effect of fucoidan on α–glucosidase was investigated using varying concentrations (ranging from 0.01 to 1000 µg/ml) of three different types of fucoidan (*Undaria Pinnatifida*, *Fucus Vesculosus*, *Fucus Synergy*). Enzyme (0.04U ml^{-1}, 50 µl) was mixed with 20 µl of each concentration of inhibitor in eppendorf tube and incubated for 10 minutes at 37 °C and 500 rpm. Technically, various concentrations of inhibitor were diluted from a stock solution (7 mg ml^{-1}) which provided 1000 µg/ml of fucoidan or acarbose in the final reaction mixture (140 µl). The fucoidan powder (70 mg) was placed in a 10 ml volumetric flask and then was sonicated using ultrasonic bath until all particles dissolved. After this, the stock solution was diluted to obtain different concentrations (0.05, 0.1, 1, 2.5, 7.5, 10, 50, 100, 500, 1000 µg ml^{-1}) of fucoidan. The preparation of positive control (acarbose) followed a similar pattern, whereas Milli–Q water was used instead of tested sample in the negative controls. Enzyme (0.04U/ml) and its activity were obtained based on the process of extraction of mammalian α–glucosidase enzyme and determination of enzyme activity respectively. A solution (n=3) containing enzyme extract and different concentrations of fucoidan was incubated at 37°C for 10 minutes. PNPG (1 mM, 20 µl) was added to the

enzyme–fucoidan mixture to initiate the enzyme–substrate reaction. The mixture was then incubated for 30 minutes at 37°C in a mixing block. Sodium bicarbonate (50 µl of 0.1M) was added to stop the enzyme–substrate reaction. At end of assay, the intensity of the colour was measured at 405 nm using a microtiter plate reader. The inhibitory effect of fucoidan and positive control were calculated using a following formula:

$$\%\,\text{inhibition} = \frac{MC - MS}{MC} \times 100$$

where MC and MS are mass of product converted in the negative control and that of sample or the positive control respectively.

Results

Enzyme activity of α–glucosidase

Enzyme activities of four different extracts were found to be 0.0076, 0.0098, 0.0237 and 0.0381 U ml^{-1}. The fourth enzyme extract (0.0381 U ml^{-1}) was used for the enzyme inhibitory assay.

Enzyme inhibitory assay of α–glucosidase

Three different extracts of fucoidan assessed in enzyme inhibitory assay had negligible effect when compared with acarbose. The maximum percentage of inhibition of acarbose was 61.11%, achieved at a concentration of 1000 μg ml^{-1}, and showed a consistent tendency during the enzyme inhibitory assay. In contrast, the maximum percentage of inhibition of fucoidan extracted from *Fucus Synergy* was 7.57%, followed by the extracts of *Fucus vesiculosus* (6.14%) *and Undaria pinnatifida* (3.30%) (Figure 1). As fucoidan did not show increase in inhibition with increase in concentration of it, it can be confirmed that all the tested fucoidan extracts did not show inhibitory effect on the enzyme.

V_{max} and K_m of α–glucosidase

The enzyme kinetics study showed that the enzyme had Km and Vmax values of 8.8339 μg ml^{-1} and 0.2885 μg min^{-1} respectively (Figure 2).

Discussion

Although the inhibitory effect of fucoidan from two commercial seaweeds (*Fucus vesiculosus* and *Ascophyllum nodosum*) on yeast α–glucosidase has been reported in 2012 (19), this study re-assessed its anti-hyperglycemic effect on the same enzyme for three main reasons. First of all, yeast α–glucosidase instead of mammalian source was used in previous study (19). The second reason to revisit is they have used four concentrations of fucoidan ranging from 0.005 to 0.05 μg ml^{-1} in 5 ml of final enzyme–substrate–inhibitor solution where as higher concentration of fucoidan is included in my experiment. Moreover they used negative control only but both negative and positive controls were used in our study. Third reason is that the activity of fucoidan was not investigated in duplicate or triplicate. Multiple analysis of the same sample is important for the reliability of the results (20).

Another study in 2012 showed that fucoidan extract from Fucus vesiculosus showed the maximum percentage inhibition of 51.4% (19), whereas fucoidan from same source and other two sources did not exhibit inhibition on the same enzyme in our study. This could be due to property of fucoidan shows highly species–specific characteristics and some other parameters such as harvesting period and growth conditions also contribute to variance on its inhibitory effect (13).

The extraction of enzyme was influenced by sonication time. Increase in sonication time increased the amount of enzyme that can be extracted from the rat intestinal powder, thus higher enzyme activity was obtained with longer sonication time. For example, the enzyme

activities of α–glucosidase was 0.0076 U ml^{-1} after 30 minutes of sonication time, compared to 0.0381 U ml^{-1} after 60 minutes of sonication.

Michaelis constant (K_m) in enzyme kinetics indicated the affinity of α–glucosidase to PNPG. K_m also equals to the concentration of substrate at which the reaction takes place at one half of its maximum reaction rate and this value is reversely proportional to the affinity (21). In this study, K_m value indicated the required concentration of PNPG and the reaction time to achieve equilibrium. Relevant studies on mammalian α–glucosidase are quite limited. However, one study in 1983 showed K_m and V_{max} values of yeast α–glucosidase from *Lactobacillus acidophilus* on PNPG as 7.9988 μg ml^{-1} and 0.001807 μg min^{-1} respectively after 30 minutes reaction (22). My experimental result showed that K_m value was similar to yeast α–glucosidase but Vmax was 160 times higher than it. That means yeast α–glucosidase and mammalian α–glucosidase have similar affinity to PNPG, whereas the rate of transformation from substrate to product by using rat α–glucosidase is 160 times more than that of yeast α–glucosidase. Additionally, according to the K_m value, the concentration of PNPG at which the reaction takes place at half of V_{max} is 0.0635 μM; therefore, only approximate 0.127 μM of PNPG is required to achieve the V_{max}, compared with 1 mM of PNPG employed in this study.

However, due to time constrain it was not possible to investigate the activity of fucoidan extracted from other sources. Also, this study did not investigate the influence of various parameters such as seasonal variation and extraction procedures on the inhibitory effect of fucoidan.

Conclusions

The inhibitory effects of three types of fucoidan were negligible, compare with acarbose, which reached a maximum percentage inhibition of 61% at 30 minutes reaction time using 1000 μg ml^{-1} acarbose. The V_{max} and K_m values of rat α–glucosidase were 0.2885 μg min^{-1} and 8.8339 μg ml^{-1} respectively. Enzyme activity of α–glucosidase was 0.0381 U ml^{-1}. According to the discussion above, α–glucosidase has high affinity to PNPG. The concentration of PNPG (1 mM) and the reaction time (30 minutes) were sufficient to achieve the V_{max}.

Declaration of interest

The author has declared no potential conflicts of interest.

List of figures

Figure 1

The inhibitory effect of fucoidan extracted from *Fucus synergy*, *Undaria pinnatifida* and *Fucus vesculosus* on the activity of α–glucosidase, in comparison with acarbose at the same concentrations

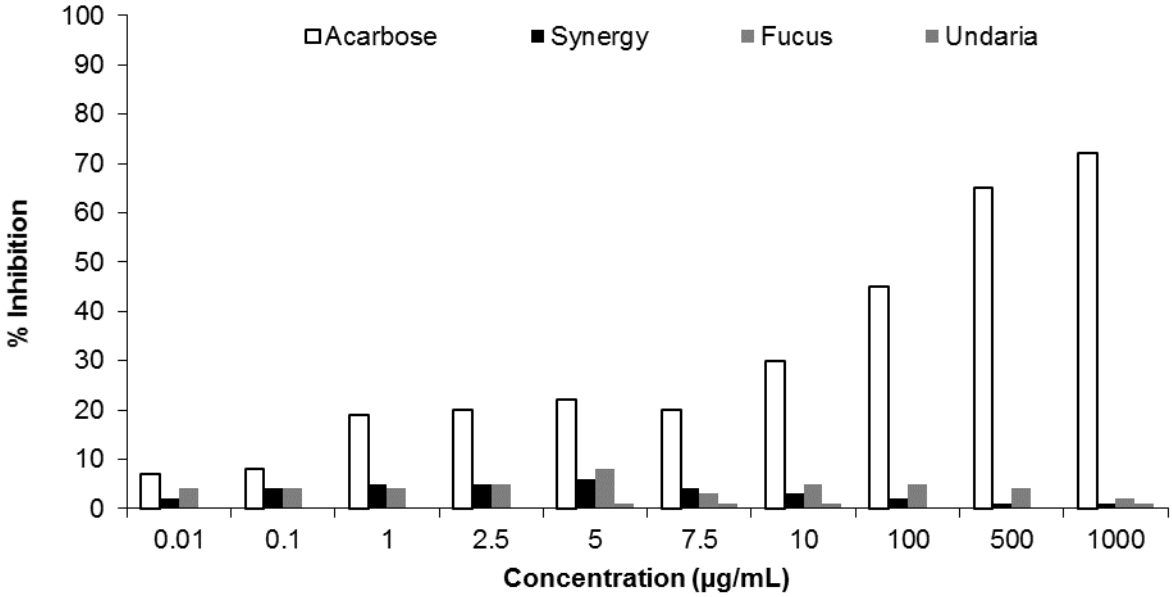
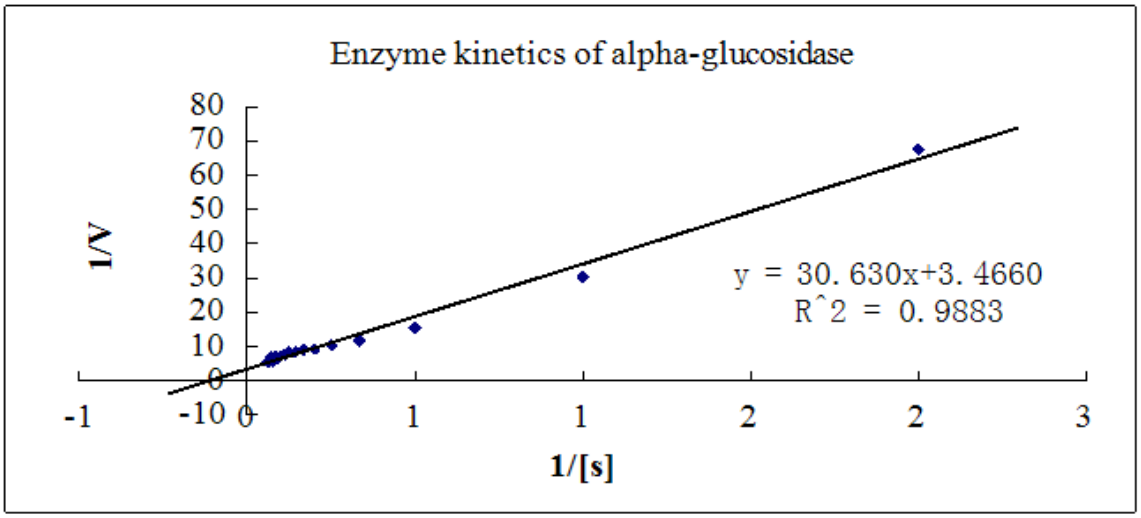


Figure 2

Partial determination of enzyme kinetics of α–glucosidase using different concentrations of PNPG (ranging from 0.5 to 16 μg ml^{-1}). The reciprocal values of intercept of X axis and Y axis indicate V_{max} and K_m respectively.



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